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Porcine Reproductive and Respiratory Syndrome Virus Subverts Repertoire Development by Proliferation of Germline-Encoded B Cells of All Isotypes Bearing Hydrophobic Heavy Chain CDR3

John E. Butler,2* Nancy Wertz,* Patrick Weber,* and Kelly M. Lager†

Isolator piglets infected with porcine reproductive and respiratory syndrome virus (PRRSV), which is related to the lactate dehydrogenase-elevating virus of mice, develop severe hypergammaglobulinemia, lymph node adenopathy, and autoimmunity. Many of the polyclonally activated B cell clones bear hydrophobic H chain CDR3s (HCDR3s) and are disseminated to most lymphoid tissues. We show in this study that B cells with identical hydrophobic HCDR3s are expressed with all major isotypes in PRRSV-infected piglets (PIPs), explaining why PRRSV-induced hypergammaglobulinemia is seen in all major isotypes. Up to one-third of randomly selected VDJ clones from the respiratory tract of PIPs have hydrophobic HCDR3s exclusively bearing VDJ rearrangements with CDR1, CDR2, and nearly intact DH segments in germline configuration. These HCDR3s are long and D_{\mu}A and D_{\mu}B are exclusively used in reading frame 3. A minimal tripeptide motif containing three hydrophobic amino acids (Leu, Val, and Ile) or any two plus alanine is common to this hydrophobic patch. We propose that PRRSV infection causes generalized Ag-independent B cell activation and hypergammaglobulinemia with biased expansion of a subpopulation of the preimmune repertoire with hydrophobic binding sites that normally disappears during Ag-driven repertoire diversification. Elevated Ig levels in PIP cannot be explained as antiviral Abs; some IgGs can account for autoantibodies to dsDNA and Golgi, whereas those with hydrophobic binding sites may account for the Ig aggregates seen in PIPs and lactate dehydrogenase-elevating virus-infected mice. This diversion from normal repertoire development may explain the delayed immune response to PRRSV. The Journal of Immunology, 2008, 180: 2347–2356.

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3 Abbreviations used in this paper: PRRS, porcine reproductive and respiratory syndrome; BAL, bronchoalveolar lavage; BSAg, B cell superantigen; dpi, days postinfection; HCDR3, H chain CDR3; H.I., hydropathicity index; HP, SIV-infected piglets; LDV, lactate dehydrogenase-elevating virus; PCV-2, porcine circovirus-2; PIC, parasite-infected conventional pigs; PIP, PRRSV-infected piglets; pnt, polynucleotide; PRRSV, virus; RF, reading frame; SHM, somatic hypermutation; SIV, swine influenza virus.

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113-day gravid outbred swine, placed in groups of four in rigid tub isolators, and reared on ESPPlac (PetAg) as previously described (5, 11). All studies were approved by the Animal Care and Use Committee of the NADC. Groups of 3–5 pigs from the same litter were maintained germ-free and treated one of three ways: 1) inoculated intranasally with 10⁴ TCID₅₀ PRRSV (where TCID₅₀ is 50% tissue culture-infective dose); 2) inoculated intranasally with 10³ TCID₅₀ SIV; or 3) given a sham inoculum. All inoculations took place on day 7 (0 days postincubation (dpi)). Animals were maintained for up to 46 days postinoculation (dpi) but most were necropsied at 10 and 25 dpi. SIV-infected (Ia30 strain) animals develop pronounced lung lesions at 10 dpi, but these are resolved on or before 25 dpi. These animals mount virus-neutralizing Ab responses (12) and are considered to have developed sterilizing immunity. The isolator piglet model is reviewed elsewhere (13, 14). A number of piglets were euthanized at delivery to obtain baseline data on the preimmune repertoire of newborns. Mesenteric lymph nodes and PBMCs from parasite-infected conventional (PIC) young pigs were kindly provided by Dr. J. Urban, Jr, U. S. Department of Agriculture, Agricultural Research Service, Beltsville, MD.

Collection of samples

Isolator piglets were euthanized with pentobarbital (Sleepaway; Fort Dodge Laboratories). At necropsy, lungs were lavaged with 50 ml of saline. Because both PRRSV and SIV are respiratory pathogens, focus was on the lung and associated lymph nodes. This bronchoalveolar lavage (BAL) was centrifuged at 1000 × g for 15 min to recover the cellular function and the supernatant was later used for Ig and protein analysis (Fig. 1). In PIPS, 2.1 × 10⁷ B cells were routinely recovered from BAL and 5.9 × 10⁶ from the BAL of IIP. The BAL cell pellet was suspended in saline for flow cytometric studies and the remainder was stored in TRizol at −20°C. Blood collected in anti-coagulation tubes was processed as previously described for the recovery of PBMCs and plasma (5, 15). The plasma was used for Ig determinations (Fig. 1) and was cultured for virus as described previously (12). Also at necropsy, various solid lymphoid tissues such as tracheal bronchial lymph nodes were also collected and frozen in liquid nitrogen.

Experimental design and VDJ cloning

VDJs were cloned to test two separate hypotheses: first to test whether in the same tissue the same clones were expressed with IgM, IgG, and IgA, and second to determine the frequency of VDJ clones bearing hydrophobic HCDR3 through random sampling. For both studies solid tissues preserved at −70°C and BAL cells in TRizol were processed for the preparation of total RNA and subsequently cDNA (5, 16). In BAL from PIPS the cDNA used in PCR represented 4 × 10⁷ B cells. VDJ clones expressed on IgM, IgA, and IgG transcripts were recovered using appropriate primer sets as described previously (17–19). These VDJs were then cloned, individual clones were grown out in microtiter wells, and their plasmid DNA was purified and then immobilized on nylon membranes. The V₄ usage of each clone was determined by sequential hybridization with probes specific for seven V₄ genes that account for >95% of the preimmune repertoire (7). “Other” V₄ means a V₄-containing clone that does not hybridize with CDR-specific probes for the seven V₄ genes; mostly because of somatic hypermutation (SHM) (7). Newborn piglets, which express a Nond diversified preimmune repertoire, and PIC pigs, which give strong Th₂ responses, were included for comparison.

Spectratypic analysis of clones of different isotypes

Only a single IgA (20), seven IgG genes, and two D₄ genes comprise 80% of the swine preimmune repertoire (7, 16). Because of this limited combinatorial diversity, the BCR repertoire is overwhelmingly determined by HCDR3 diversity (21). Diversity in HCDR3 lengths is a quasi-clone marker, so spectratypic analysis (HCDR3 length analysis) provides a quasi-clonotypic analysis (6, 22). The HCDR3 segments from VDJ clones expressed with IgA, IgM, and IgG from the same tissue sample from the same animal were recovered by PCR and the products were spectratyped side-by-side so that the profiles for IgA, IgM, and IgG could be compared. Same-length HCDR3 polynucleotides (pnt) shared among isotypes were recovered from polycrylamide spectratyping gel segments using a scalpel and placed into 100 μl of ddH₂O (6). These were incubated overnight to permit diffusion of the DNA and the fluid phase was targeted for 15 cycles of PCR with the primers used for the original amplification of HCDR3. These HCDR3s were cloned and sequenced.

The frequency and analysis of VDJ clones

In a previous study (6) we analyzed only HCDR3s from clones expanded in PRRS. In this study we randomly selected approximately equal numbers of VDJ clones using V₄-, V₅-, and “other” V₆ genes that were expressed with IgM, IgA, and IgG (Table I). These three gene categories account for 55–75% of all V₄ usage in PIPS. DNA from selected clones was amplified, products of the expected length were verified by agarose gel electrophoresis, and the gel segments were recovered and cloned into pCR2 TOPO (Invitrogen Life Technologies). Plasmid DNA was sequenced using the four-color ABI PRISM DNA analyzer (Applied Biosystems). Nucleotide sequences were analyzed for V₄, D₄, and SHM using the Omiga program (Accelrys). The deduced amino acid sequence of the HCDR3 region, extending from but not including the framework 3 cysteine at position 104, down to the 5′ region of J₄, but without including the invariant tryptophan that starts framework 4, were analyzed as described by Kyte and Doolittle (23) using the principles described by Eisenberg (24) as applied to HCDR3 sequences by Ivanov et al. (9). Sequence data were used to compute a hydropathicity index (H.I.). Based on inspection of sequences and calculations of H.I., it became clear that HCDR3s dominated by LV, I, M, and A and without R, K, and D would have H.I. > 5.0, whereas those dominated by R, D, K, and H and would be strongly hydrophilic (H.I. = −0.4 to −0.1). Sequences containing mixtures of these amino acids and those of neutral charge belong to region II (0.0–0.3). HCDR3 sequences from randomly selected VDJ clones of known V₄ and isotype usage from PIPS and IIPs were compared with HCDR3s of randomly selected VDJ clones from newborn piglets and PIC young pigs (6, 7).

### Table I. The distribution of VDJ clones selected for study

<table>
<thead>
<tr>
<th>Isotype Used</th>
<th>V₄Ψ</th>
<th>V₅Ψ</th>
<th>Other</th>
<th>Total Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>IgG</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>IgM</td>
<td>11</td>
<td>9</td>
<td>7</td>
<td>27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isotype Used</th>
<th>V₄Ψ</th>
<th>V₅Ψ</th>
<th>Other</th>
<th>Total Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>IgG</td>
<td>6</td>
<td>9</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>IgM</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>21</td>
</tr>
</tbody>
</table>

*Sequence analyses revealed that, in the case of PRRS, all clones picked as “other” and with hydrophobic HCDR3s were V₄Ψ clones that, for unexplained reasons, did not originally hybridize for both V₄Ψ-specific CDR1 and CDR2 probes.*

### Determination of total Ig levels

IgM, IgA, and IgG levels in blood plasma and BAL were determined by sandwich ELISA as previously described (25).

### Statistical analysis

Simple mean differences were compared by two-sided Student’s t analysis using the stats program of Prism (GraphPad Software). Statistical analyses of HCDR3 distribution among hydropathicity regions was done with the help of Dr. K. Chaloner (Head of the Department of Biostatistics, University of Iowa College of Public Health, Iowa City, IA) using the method of Holm (26), and exact tests were implemented by the R Development Core Team of Vienna Austria (www.R-project.org).

### Results

Ig levels are greatly elevated in blood and BAL in PRRS

Fig. 1 shows that plasma IgM, IgG, and IgA are elevated >10-fold in PIPS compared with those infected with SIV and porcine circovirus-2 (PCV-2). Thus, only PRRSV induces the hypergamma-globulinemia we have previously described (5), indicating that it is specific to PRRSV and not a generic effect of the viral infection of isolator piglets. The kinetics of the hypergamma-globulinemia differs among isotypes. IgM levels are elevated at 10 dpi but serum IgG and IgA levels peak at 25 dpi. Significantly elevated levels are...
also present in the BAL fluid of PIPs, although peak levels of all Igs are maintained longer in BAL (e.g., 46 dpi).

**B cells expressing different isotypes in PIPs express the same hydrophobic HCDR3s**

Fig. 2 demonstrates that the Gaussian-like polyclonal HCDR3 length profile of bone marrow shows evidence of selection in the mesenteric lymph nodes of conventional control pigs. In PIPs, the quasi-oligoclonal IgG spectratype is shared by nearly all tissues including those remote from the site of infection (Fig. 2 and Ref. 6). Fig. 3, *left panel*, shows that an oligoclonal pattern shared among isotypes in PIPs begins to appear at 10 dpi but does not spread to all isotypes until 25 dpi. At that time IgG, IgM, and IgA display nearly identical oligoclonal HCDR3 length profiles. This pattern of shared same-length HCDR3s is sometimes seen out to 46 dpi (Fig. 3, *lower left panel*). When isotype HCDR3 profiles from IIPs and sham-inoculated controls were studied the pattern is also quasi-oligoclonal but, with few exceptions, IgG, IgM, and IgA do not display the same profile as that seen in PIPs (Fig. 3B).

Sharing the same profile among spectratypes generated from different isotypes only suggests shared clonality, because many different B cell clones can have HCDR3s of the same length. This assumption was tested by the recovery of prominent HCDR3s of 60 pnt in length that were shared by all three isotypes (Fig. 3, *left panel*; vertical bar and star). These were cloned and sequenced as previously described (6). We also choose same-length HCDR3s that were prominent and shared among isotypes in IIPs (42 pnt) and those that fit the same criterion for sham-inoculated animals (36 pnt). The results of analyzing a dozen clones of each isotype for each treatment group are summarized in Table II. Those recovered from PIPs differ from controls (IIPs and sham) in several ways. Most obvious is the use of $D_{HA}$ in reading frame (RF) 3 (65% for PIPs vs 15% for IIP and sham). Overall use of $D_{HA}$ RF3 plus $D_{HB}$ RF3 was significantly greater in PIPs (74% vs 67% vs 33% for PIPs, IIP, and sham, respectively; $p < 0.01$). Usage of RF3 in PIPs was heavily skewed to favor $D_{HA}$ over $D_{HB}$. Although the majority of DH usage was RF3 in PIPs, the majority of VDJ in IIPs and sham controls used $D_{HA}$ and $D_{HB}$ in RF2 (data not shown).

A high proportion of duplicate clones were recovered in IgA and IgG transcripts from PIPs and from all isotypes in IIPs and sham controls (Table II). Notably, half of the unique IgA and IgG clones in PIPs were common to both, and some of these were also found in IgM transcripts. By contrast, this was not seen in IIPs and only once in sham animals. We interpret this to mean...
that: 1) 30–50% of same-length HCDR3s from all treatment groups come from the same B cell clone; but 2) only in PIPs are 30% of these shared among isotypes.

**PIPs and newborns have many VDJs with hydrophobic HCDR3s**

One-third of randomly selected VDJ clones from newborns (pre-immune repertoire) comprise hydropathicity region I (H.I. \(0.5–0.8\)) whereas after Ag exposure, e.g., PIC young pigs, there is nearly complete loss of region I and a significant increase in region III (Fig. 4A). Approximately 40% of VDI clones randomly selected from PIP also appear in region I, which is significantly greater than the percentage for randomly selected IIP clones (Fig. 4B). The profile for IIP resembles that of PIC pigs. Approximately 40% of VDJ clones randomly selected from PIP also appear in region I, which is significantly greater than the percentage for randomly selected IIP clones (Fig. 4B).

**Region II of PIP shows normal repertoire diversification**

Half of the sequences in region II for both PIP (14/32) and IIP (17/39) use “other” \(V_H\) and, of these, half are somatically mutated (data not shown). Region II encompasses the predominant hydropathicity region for HCDR3s from all of the piglets studied (Fig. 4), which is the same for mice and humans (7, 8, 9). Use of “other” \(V_H\) is a feature of repertoire diversification and in young PIC pigs it comprises 80% (10), whereas only 15% of the pre-immune repertoire of newborn uses “other” \(V_H\) (10, 16, 21). Therefore, both IIP and PIP have undergone repertoire diversification but mostly in region II.

**VDJs with hydrophobic HCDR3s from PIPs are especially pronounced in IgM transcripts**

Sixty percent of randomly selected IgM clones from PIPs have hydrophobic HCDR3s (region I; H.I. \(0.5–0.8\)), which is significantly greater than that in IIP littermates (Fig. 4A). The hydropathicity profile of HCDR3 recovered from IgG transcripts of PIPs was also significantly shifted to the hydrophobic region (38%) in comparison to that of IIPs (8%; Fig. 5B), although this shift was not seen in the IgA transcripts from PIP (Fig. 5C).

**Hydrophobic HCDR3s in PIPs are especially expressed with VHZ but not with “other” \(V_H\)**

\(V_H\) usage in PIPs failed to indicate a preferential \(V_H\) usage associated with this infection (6, 27), thus failing to provide evidence."
for a conventional B cell superantigen (BSAg) effect (28, 29). In all newborn piglets, V_{H}A expressed with IgM and IgG comprises 25–35% of total V_{H} usage (Table III), but “other V_{H}” contributes up to 45% in PIPs (6, 27). More recent studies have shown that V_{H}Z accounts for 10% of all V_{H} usage (Table III) and that its usage doubles in PIPs (10–20%) while V_{H}B usage sharply declines (Ref. 27 and J. E. Butler, P. Weber, and N. Wertz, unpublished observations). Therefore, we chose to focus on the three V_{H} categories that account for 55–70% of the total repertoire in PIPs: V_{H}A, V_{H}Z, and “other V_{H}”. Fig. 6 shows that the hydrophobic HCDR3 population (region I) expressed with V_{H}Z is significantly over-represented (57%) and that only one “other” V_{H} clone appears in this category. This is a nonmutated V_{H}X (IgG 49E4; Table IV). Although hydrophobic HCDR3s are also expressed with V_{H}A, the proportional usage of V_{H}A does not differ from its usage in IIPs.

**Hydrophobic HCDR3s are long and rarely show SHM**

A total of 25 VDJ clones account for all the hydrophobic HCDR3s in region I (0.5–0.8) in PIPs (Fig. 4B; Table IV). When the CDR1 and CDR2 regions of all 25 VDJ clones with hydrophobic HCDR3s were examined, one used an “other” V_{H} (see above) and all but three were in germline configuration, but these carried silent mutations (IgM F5, IgG F10, IgG F3).

Furthermore, ~70% of the D_{H} regions remained in germline configuration although truncated to some degree. More than 70% of these VDJs use D_{H}A and more than half are expressed with IgM, consistent with the pattern observed when sequences were displayed according to isotype (Fig. 5A). It is noteworthy that there are no hydrophobic HCDR3s that were not encoded by RF3 of D_{H}A or D_{H}B, indicating either that: 1) any hydrophobic sequence cannot substitute for those encoded especially by D_{H}A; or 2) hydrophobic HCDR3s not encoded by RF3 of D_{H}A or D_{H}B are so rare in infected isolator piglets that the chance of their recovery from 70 sequences is remote. Although several related tripeptide motifs, Leu-Val-Ala, Ile-Ala-Val, Val-Ala-Val, are encoded by both D_{H}A and D_{H}B, there are so few D_{H}A RF 3 clones were recovered from IIP and sham to allow a comparison.

### Table II. Characteristic of clones from same length HCDR3s expressed with IgM, IgG, and IgA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PIPs</th>
<th>IIPs</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Igs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Identification</strong></td>
<td>IgM</td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>Length of germine</td>
<td>7.4 ± 1.9</td>
<td>7.2 ± 2.7</td>
<td>6.4 ± 1.9</td>
</tr>
<tr>
<td>Length of germine</td>
<td>5.0 ± 0</td>
<td>7.3 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Shared with other isotypes</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Ids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Identification</strong></td>
<td>IgM</td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>Length of germine</td>
<td>5.0 ± 0</td>
<td>6.0</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>Shared with other isotypes</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**a** The proportion of identical clones to the total; e.g., in the case of IgG, 5/13 share one sequence, 2/13 share another, and still 2/13 share another sequence. Clones that differ by one nucleotide are considered identical. Of the total, only 2.6% had a one-nucleotide change.

**b** D_{H}A in RF3 encodes 11 amino acids and D_{H}B in RF3 encodes eight amino acids. In PIP, 64% of D_{H}A is full length. Too few D_{H}A RF 3 clones were recovered from IIP and sham to allow a comparison.

**c** Not applicable since there are no clones.

Bias for selective expansion of B cells in PIPs with hydrophobic HCDR3s is not augmented by the hydrophobicity of CDR1 and CDR2

V_{H}Z usage is increased in PRRSV infections (J. E. Butler, P. Weber, and N. Wertz, unpublished observations) and significantly
over-represented in region I (Fig. 6B). This is correlated with the relatively high hydrophobicity of CDR1 of VHZ (Table III). However, if the hydrophobicity of CDR1 and/or CDR2 is important, clones expressing VHF, VHY, and perhaps VHE should also have shown increased usage in PIPs. Although this is not seen, the increase in VHA at 10 dpi is counteracted by a decrease in VHB usage, which is the most hydrophilic VH gene of the porcine preimmune repertoire. (Table III). Thus, the hydrophobicity of at least CDR1 may contribute to VH selection, although the hydrophobicity of HCDR3 appears to be the major determinant in selective expansion.

Discussion
The data presented extend in five ways our initial observation concerning the expansion, dissemination, and differentiation of B cells in PIPs with hydrophobic HCDR3s (6). First, we show that the hypergammaglobulinemia in PIP is not a generic effect of the viral exposure of isolator piglets to any virus and that elevated Ig levels are also seen at the site of infection (BAL; Fig. 1).

Second, we show that by 25 dpi all major isotypes in PIPs show selective expansion of HCDR3s derived from the same parent B cell clones (Fig. 3, left panel, and Table II). This explains why the hypergammaglobulinemia in PIPs affects all major isotypes in both blood and BAL (Fig. 1). Because fetal piglets undergo class switch

Table III. The protein sequence and H.I. of CDRs of the major VH genes comprising the preimmune repertoire in swine

<table>
<thead>
<tr>
<th>VH Gene</th>
<th>Percentage Usage (%)</th>
<th>Sequence (H.I.)</th>
<th>CDR1</th>
<th>CDR2</th>
<th>Combined H.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>STYIN (0.03)</td>
<td>AISTSGC (0.33)</td>
<td>0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VHE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>DNAFX (0.03)</td>
<td>AIASSDYDG (0.11)</td>
<td>0.067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VHE</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>SYEIS (0.03)</td>
<td>GIYSSGYS (0.15)</td>
<td>0.040</td>
<td></td>
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<tr>
<td>VHE</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td>SYEIS (0.03)</td>
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<tr>
<td>VHE</td>
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<td>SYEIS (0.03)</td>
<td>AISTSGC (0.48)</td>
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<td>VHE</td>
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</tr>
<tr>
<td>10</td>
<td>SYEIS (0.03)</td>
<td>GIYSSGYS (0.15)</td>
<td>0.250</td>
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<td></td>
</tr>
</tbody>
</table>

a Mean values for newborn piglets.

b Not applicable.
recombination without SHM halfway through gestation in the absence of environmental Ag, possibly stochastically (18, 30, 31), the association of a germline (preimmune) repertoire with IgG and IgA as well as IgM is not surprising.

Third, we show that 30–40% of randomly selected VDJ clones from PIP belong to hydropathicity region I, which nearly disappears in PIC pigs and IIP littermates (Fig. 4). A similar shift is also seen in all mouse B cell subsets after the early transitional (T1) stage (9). The disappearance of region I following Ag exposure may result from death by neglect, because these HCDR3s have been considered least optimal for paratopes recognizing pathogens (32). HCDR3s in region I express a nonmutated germline VH repertoire, retain >70% of their germline D_{pA} and D_{pB} sequences, all of which are expressed in RF3, and these HCDR3s are significantly longer than the mean length of the preimmune repertoire (Table IV). The higher incidence of IgM clones with BCRs bearing nondiversified hydrophobic HCDR3s (Fig. 5) is reminiscent of the preimmune repertoire in which IgM dominates (18). Additional support for the preimmune nature of the expanded subpopulation comes from cyometric studies of B cells from the BAL of PIPs, showing that a very high proportion are CD2^{+} compared with IIP littermates; CD2 in swine is a marker for undifferentiated B cells (33). The shift away from B cells with hydrophobic BCRs after Ag exposure correlates with an increase in use of “other” V_{H} genes from 15 to 85%, in PIC pigs (10) “Other” V_{H} usage also occurs in PIP and IIP but only in region II (Fig. 4B).

Fourth, because approximately one-third of the B cells from BAL in PIPs belong to region I (Fig. 4B) and many tissues share the same spectratype (Fig. 2), it suggests that the same B cell subpopulation is disseminated to all lymphoid tissues (Fig. 2 and Ref. 6). A 4-wk-old piglet has 3 \times 10^{11} lymphocytes (34) although isolator piglets may have half that number, of which 16% of the PBMCs are B cells (35). If one-third of all B cells in PRRS comprises the expanded preimmune repertoire (Table IV), it suggests that there may be 8 \times 10^{7} cells with hydrophobic, germline-encoded BCRs in PIP. The number of such cells approaches the number of malignant B cells in the bone marrow of myeloma patients (N. Rosenthal (Department of Pathology, University of Iowa, Iowa City, IA), unpublished observations). Furthermore there would be more serum IgG with hydrophobic binding sites (15 mg/ml) than the total IgG concentration in PIC young pigs (Fig. 4C). Finally, this study better defines the motif in the BCR of expanded B cells in PIPs. The minimal motif appears to be a tripeptide rich in isoleucine, valine, and leucine often in association with an alanine. A pentapeptide motif (AMVLV) is encoded by D_{pA}, but the tripeptide is common to D_{pA} and D_{pB} (Table IV). Adjacent hydrophobic amino acids may also contribute to a hydrophobic patch, which may explain the preference for D_{pA} and V_{H}Z with its hydrophobic CDR1 (Table III and Fig. 6). In addition, the significantly longer HCDR3s in region I of PIP (p < 0.01) compared with the mean HCDR3 length in the preimmune repertoire (Table IV and Ref. 21) would increase the size of the patch. The longer HCDR3 is not the result of an age-related increase in terminal deoxynucleotidyltransferase (TdT) activity, because TdT is active at the first time of VDJ rearrangement at 20 days of gestation in the yolk sac (36) and there is no change in mean HCDR3 length throughout fetal life (21). Hence, HCDR3 length, hydrophobicity and even V_{H} usage may contribute to the formation of a hydrophobic patch.

PRRSV is a member of the Arteriviridae that includes equine arterivirus, simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus (LDV) of mice. The latter is the best studied and is persistent for life (37). The appearance of inefficient neutralizing Abs is delayed, appearing 1–2 mo after infection (38). LDV produces polyclonal B cell activation (39, 40) that results in the formation of immune complexes and autoantibodies (41, 42). These have been reported not to contain anti-LDV Abs but appear to be complexes of autoantibodies and self-antigens (43) and hydrophobic aggregates that spontaneously adsorb to polystyrene (44). Thus, generalized polyclonal B cell activation characteristic of PRRSV and LDV could generate autoantibodies to dsDNA, Golgi glycoproteins, and other autoantigens. The immune response to PRRSV is also characterized by a delay in responsiveness (45, 46), a surprisingly weak inflammatory response in the lung (47, 48), and the need for 150 days to clear the infection (4). This delay is believed to increase susceptibility to secondary infections that may also interfere with the effectiveness of vaccines (49–51). Thus, there are numerous features of the response to LDV that parallel those to PRRSV, although studies at the molecular level on LDV Abs like those described in this article have not been reported. LDV is a persistent infection that causes no harm, whereas PRRSV is responsible for a world pandemic in swine (1). The phenomenon we describe for PIP contains a nearly untestable but reasonable assumption, namely that one-third of the 10- to 100-fold increase in Igs, i.e., 15 mg/ml plasma IgG (Fig. 1), is derived from B cells bearing hydrophobic motifs (Fig. 4C). Because the response is polyclonal, individual Igs cannot be recovered for sequencing to test this assumption unless they can be.
Characteristics of 25 VDJ s in region I with hydrophobic HCDR3 s from PIPs

<table>
<thead>
<tr>
<th>Clone</th>
<th>V H Gene</th>
<th>H.I.</th>
<th>D H Usage</th>
<th>FR3</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>IgG A4</td>
<td>V µ A</td>
<td>0.59</td>
<td>A</td>
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<td>3</td>
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<td>0.66</td>
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<td>8/11</td>
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<td>V µ Z</td>
<td>0.62</td>
<td>A</td>
<td>3</td>
<td>8/11</td>
</tr>
<tr>
<td>IgA B9</td>
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<td>A</td>
<td>3</td>
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<tr>
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</tr>
<tr>
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<td>V µ Z</td>
<td>0.50</td>
<td>A</td>
<td>3</td>
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</tr>
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</tr>
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</table>

GenBank accession numbers for the clones are EU267244–EU267268.

Note: HCDR3 region is separated into distinguishable parts. The regions 5′ and 3′ of D H do not distinguish among n-region additions, palindromic additions, or somatic hypermutation of D H and J H gene segments. The mean HCDR3 length for these sequences is 55.0 ± 11.5 pnt, which is significantly longer (p < 0.01) than the mean of 41.2 ± 9.2 for all pre-immune HCDR3 (21).

Transformed and cloned. Accepting this assumption, the elevated level of IgG with hydrophobic binding sites could contain specific Abs to a hydrophobic epitope of a T cell-independent (TI) Ag from the viral envelope (52) or the amphipathic helix of the M protein (53). These may have been overlooked because commercial and experimental ELISAs depend on hydrophilic glycoproteins or nucleocapsid proteins (see below) and because ELISAs are biased against hydrophobic Abs (54). Finding such a high level of specific Abs in viral infections would be quite extraordinary. Initially we showed that <1 of the total IgG recognized PRRSV Ags using the HerdChek assay (IDEXX Laboratories). Dot blot assays using inactivated whole virus gave similar results (27). There is some evidence for autoantibodies to hydrophobic self-antigens, e.g., those to TGFβ in lpr mice (55) and perhaps in LDV (44). Sixty percent of IgGs in hypergammaglobulinemia plasma (25 mg/ml IgG and double the concentration in PIP; Fig. 4C) have HCDR3s in hydrophilicity region II with a charged binding site. The autoantibodies to Golgi proteins, dsDNA, and others we observed (5) would most likely be accounted for by these IgGs rather than by those with hydrophobic HCDR3s (region I; Fig. 4). This assumption is based on studies showing that autoantibodies are typically rich in arginine and lysine and would distribute to region II (28, 56–58). It is generally recognized that: 1) the “natural” preimmune repertoire overexpresses autoreactive Abs (59–62); and 2) polyreactivity is associated with HCDR3 (63–65). The Abs to PRRSV glycoproteins and the highly immunogenic nucleocapsid protein in PIP (Refs. 66–72; M. Murtough, unpublished observations) are most likely to have HCDR3s that fit to region II, which is where we observed that repertoire diversification had occurred (Fig. 4B).
permissive macrophages through CD163 (85), and infectivity depends on the interaction of viral RNA with CD151 (86). Hence, such infected macrophages might present both viral RNA and an endogenous BSAg to B cells. The putative BSAg hypothesis is consistent with observations that the preimmune repertoire, such as that encoded in marginal zone and B-1 cells, is preferentially susceptible (78, 87, 88). Interestingly, conventionally reared PIPs show a reduced degree of immune dysregulation compared with isolator PIPs (27), perhaps because a much smaller proportion of nondiversified B cells remain. This may also explain why adult mice infected with LDV show a reduced level of immune dysregulation compared with isolator piglets (43).

This study raises evolutionary and biotechnical issues concerning BCRs with hydrophobic HCDR3s. If these are deselected because there are no hydrophobic Ags (32, 89), why do mammals continue to generate them as part of their preimmune repertoire? Is it possible that there are important hydrophobic epitopes that immunologists have overlooked because of the entrenched bias against hydrophobic Abs in ELISAs?

The transition from fetal life to weaning traverses a “critical window” in immunological development (13, 14) in which the neonate must: 1) juggle the protective and/or suppressive effects of passive immunity; 2) respond to the pathogen-associated molecular patterns of gut flora that stimulate development of the adaptive immune system; 3) refine the innate protective preadaptive (natural) Ab repertoire to one that is less cross-reactive, less autoreactive, and with refined specificity to pathogens; and 4) simultaneously develop tolerance to nonpathogenic foreign Ags. Failure to establish homeostasis during this process results in immune dysregulation. This “window” is when pathogens and BSAgs may have their greatest impact (90). We believe PRRSV is a pathogen that uses polyclonal B cell activation through a virus-derived or virus-stimulated BSAg (84) to subvert the normal development of adaptive immunity. This distraction causes a delay in the appearance of a protective antiviral response and, consequently, resolution of the infection. This allows more time for viral replication and subsequently a greater chance for the infection to spread to other swine.

Disclosures
The authors have no financial conflict of interest.

References


